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On-line electrodialysis–capillary zone electrophoresis of adenosine triphosphate and inositol phosphates

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Abstract

Electrodialysis (ED) has been coupled to capillary zone electrophoresis (CZE) as an on-line sample pretreatment technique. Analytes are introduced into the fused-silica capillary through a membrane with a molecular mass cut-off of 30 000. As reversed polarity is applied only small negatively charged ions are injected whereas positively charged and/or large compounds are retained. ED takes only ca. 20 s and is comparable with electrokinetic injection in several respects. ED–CZE has been applied to adenosine triphosphate in blood plasma and to inositol phosphates in fermentation broth.

Keywords: Electrodialysis; Adenosine triphosphate; Inositol phosphates

1. Introduction

After fifteen years of development, capillary zone electrophoresis (CZE) has been introduced in many (routine) analytical laboratories. For clean samples, CZE without sample pretreatment works quite well. However, real-life samples require a laborious and time-consuming pretreatment before CZE can be performed. Most of these sample pretreatment techniques are performed off-line. However, an on-line liquid–liquid electroextraction–CZE method has been developed lately which can be performed e.g. after (off-line) solid-phase extraction (SPE) [1,2]. Furthermore, an on-line SPE–CZE procedure was developed by Strausbauch et al. [3]. Unfortunately, the described procedures are not suitable for highly polar compounds such as inositol phosphates which are ionized over nearly the whole pH range.

Inositol phosphates are important compounds in several research areas [4,5]. These phosphorylated sugars are negatively charged, even at low pH. Besides, they do not contain any chromophoric/fluorophoric groups in the molecular structure which makes sensitive detection rather difficult. Analysis of inositol phosphates in complicated matrices, such as fermentation broth, blood plasma or tissue homogenate, requires a sample pretreatment before their separation. So far, samples containing inositol phosphates have been pretreated using anion-exchange SPE [6,7], ultrafiltration [8], centrifugation [9,10] or iron(III)-loaded stationary phases [11], all being rather laborious, time-consuming and off-line methods.

In order to separate low-molecular-mass analytes from macromolecular compounds in the sample, dialysis as well as microdialysis have been combined with liquid chromatography (LC) in the on-line mode [12–15]. Furthermore, several researchers

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coupled microdialysis on-line to capillary electrophoresis [16–19]. By applying a voltage over the dialysis membrane, analytes do not only diffuse but also selectively electromigrate through the membrane. This technique, which is called electrodialysis, has been used for biotechnological purposes [20–22], neutralization of samples [23–26], enrichment [27] and purification of bioanalytical [28] and environmental [28,29] samples. In comparison to dialysis, electrodialysis is much faster and more selective.

This paper describes the on-line coupling of electrodialysis to CZE for inositol phosphates. Electrodialysis has been compared with electrokinetic injection. As inositol phosphates cannot be detected with UV detection, adenosine triphosphate, with nearly the same electrophoretic mobility as inositol triphosphate, has been used as a model compound during the development.

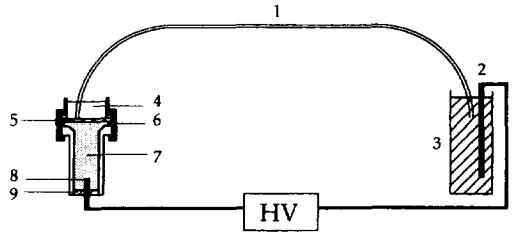
2. Experimental

2.1. Chemicals

Acetic acid p.a. was purchased from J.T. Baker (Deventer, Netherlands). Hydroxypropylmethylcellulose (HPMC), with a viscosity of 4000 cP for a 2% aqueous HPMC solution, bovine albumin (fraction V, 98–99%), inositol monophosphate (2-IP_1) as dicyclohexylammonium salt and phytic acid (IP_6) were provided by Sigma (St. Louis, MO, USA). Ammonium acetate p.a. came from Merck (Darmstadt, Germany). Adenosine 5′ triphosphate (disodium salt hydrate, 98%) and 1-naphtol-3,6-disulfonic acid (NDSA) were from Janssen (Beerse, Belgium). Inositol bis-(1,2-IP_2), tris-(1,2,6-IP_3) and tetrakis-(1,2,5,6-IP_4) phosphate were supplied as sodium salts by Perstorp Pharma (Perstorp, Sweden). Blank plasma came from the University Hospital Leiden (Leiden, Netherlands). For the preparation of the stock solutions of the analytes and buffer solutions, deionized water was used (Milli-Q system, Millipore, Bedford, MA, USA). The buffer solution was filtered through a 0.2-μm Nylon acrodisc syringe filter (Gelman Sciences, Ann Arbor, MI, USA)

2.2. Equipment and procedure

The electrodialysis–capillary zone electrophoresis set-up is shown in Fig. 1. The system consisted of two parts: an electrodialysis device (EDD) and a custom-made capillary electrophoresis (CE) system. The EDD was a modified ultrafiltration device (Amicon, Danvers, MA, USA) and consisted of a donor (7) and an acceptor (4) compartment (both 0.5 ml) separated by a membrane (5) with a molecular mass cut-off of 30 000 and a diameter of 16 mm (Amicon). A silicone O-ring (6) was positioned between the donor compartment and the membrane to prevent sample leakage along the membrane. The donor compartment was filled with sample solution and sealed with a silicone septum (9) through which



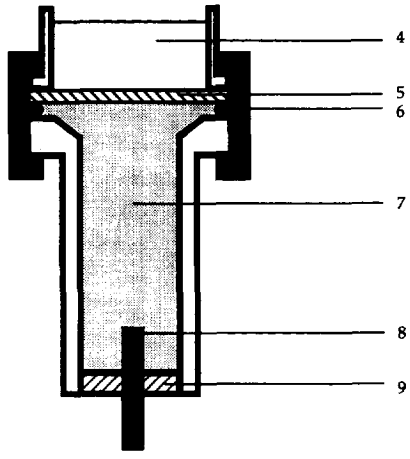


Fig. 1. Electrodialysis device coupled to the CZE system. 1 = fused-silica capillary; 2 = anode; 3 = buffer vial; 4 = acceptor compartment; 5 = membrane; 6 = O-ring; 7 = donor compartment; 8 = cathode; 9 = septum.

the platinum cathode (8) was positioned. The acceptor compartment was filled with Milli-Q water. The inlet of the fused-silica capillary (1) (SGE, Ringwood, Vic., Australia) was positioned in the acceptor compartment, the outlet of the capillary in the buffer vial (3), together with the platinum anode (2). The electrophoresis buffer consisted of 50 m*M* ammonium acetate pH 5.0 and 0.005% HPMC, freshly prepared every day. Electrodialysis was performed by applying a high voltage (Gamma High Voltage Research Inc., Mt. Vernon, NY, USA) over the EDD and fused-silica capillary (75 μm I.D., 375 μm O.D., l=0.75 m). After electrodialysis the EDD was replaced by a buffer vial and capillary zone electrophoresis was started at −25 kV (90 μA). At 0.25 m before the capillary outlet, UV detection (CE-adapted-Spectroflow 773, Kratos Analytical Instruments, Ramsey, NJ, USA) was performed at 200 nm, unless stated otherwise.

2.3. Inositol phosphates in fermentation broth

An amount of 1 ml of fermentation broth, used for the enzymatic hydrolysis of phytic acid [10], was spiked to a concentration of 100 μ*M* inositol hexakis-, tetrakis-, tris-, bis- and monophosphate. Samples were analyzed using electrodialysis (20 s, −25 kV)–CZE. For these experiments, the same set-up was used as described above. The CZE buffer consisted of 0.5 m*M* NDSA, 0.005% HPMC and 30 m*M* acetic acid pH 3. During CZE a high voltage of −30 kV (35 μA) was applied. Indirect UV detection was performed at 214 nm.

3. Results and discussion

3.1. Electrodialysis

In dialysis, solutes diffuse from one compartment to another through a membrane as a result of a concentration gradient. This implies that equilibrium is reached if the concentration gradient approaches zero. If, in conjunction with the concentration gradient, a voltage is applied over the membrane analyte ions do not only move by molecular diffusion but also by electromigration. Thus, the process, called electrodialysis, becomes much faster and more selective. Similar to dialysis, the electrodialysis set-up consists of a donor and an acceptor compartment, separated by a membrane. By positioning the cathode and the anode which are covered with ion-exchange membranes in the separate compartments, only the ions with the appropriate charge and with a molecular mass smaller than the membrane cut-off value will migrate into the acceptor compartment. (Electro)dialysis can be performed in the static or in the dynamic mode. In the static mode the donor and acceptor phase are stagnant whereas in the dynamic mode at least one of the phases is moving. Consequently, enrichment of the sample solution by dialysis can be obtained only in the dynamic mode. By electrodialysis, however, analyte concentration can be achieved in the static as well as in the dynamic mode. Besides, conductivity differences between the donor and acceptor phase can also contribute to analyte enrichment.

In general, samples containing macromolecular compounds next to the analyte are pretreated using (ultra)filtration, centrifugation or (micro)dialysis prior to the separation. However, a major drawback of these sample pretreatment techniques is their duration. Furthermore, on-line performance of (ultra)filtration or centrifugation to capillary zone electrophoresis (CZE) is rather complicated. By using electrodialysis on-line to CZE, sample pretreatment and separation of the analytes becomes non-laborious and fast.

3.2. Electrodialysis–capillary zone electrophoresis

The on-line electrodialysis–capillary zone electrophoresis (ED–CZE) set-up is shown in Fig. 1. The cathode is positioned in the donor compartment and the anode in the buffer vial. Whereas during electrokinetic injection of a sample the capillary inlet is placed in the sample vial, in on-line ED–CZE the capillary inlet is positioned in the acceptor compartment. In general, the acceptor compartment is filled with water which prevents contamination of the capillary inlet with impurities from the sample. For the performance of off-line ED(–CZE), the anode can be positioned in the acceptor compartment. Electrochemical reactions of the analytes which might occur under these conditions can be avoided by covering the electrodes with ion-exchange mem-

branes [28]. In the on-line ED–CZE set-up (Fig. 1), however, electrochemical reactions of the negative analytes do not take place at the anode before detection.

The analysis of a sample using on-line ED–CZE is a two-step procedure. In the first step, negative analytes smaller than the membrane cut-off value migrate from the (stagnant) donor compartment via the (stagnant) acceptor compartment into the fused-silica capillary by applying a voltage over the whole system. At the same time, electromigration of small negative (to the anode) and positive (to the cathode) ions occurs. The EDD does not contribute significantly to the total electrical resistance of the system. This is shown by the current in the system which is constant, with or without the EDD. After the introduction of analyte ions into the capillary, the EDD is removed and CZE is performed. As adenosine triphosphate (ATP) and inositol phosphates are multiply negatively charged at pH 5, the electrophoretic mobility is quite high. An electroosmotic flow (EOF) in opposite direction would lead to a small net velocity. Therefore, the EOF is suppressed by adding hydroxypropylmethylcellulose to the electrophoresis buffer and reversed polarity is applied during ED and CZE. The result is depicted in Fig. 2. The electropherograms of pure water (A) and an ATP standard solution (B) analyzed by ED–CZE are shown. For standard solutions of ATP, electrokinetic injection and electrodialysis gave similar electropherograms; nor dilution nor concentration of the analyte occurred.

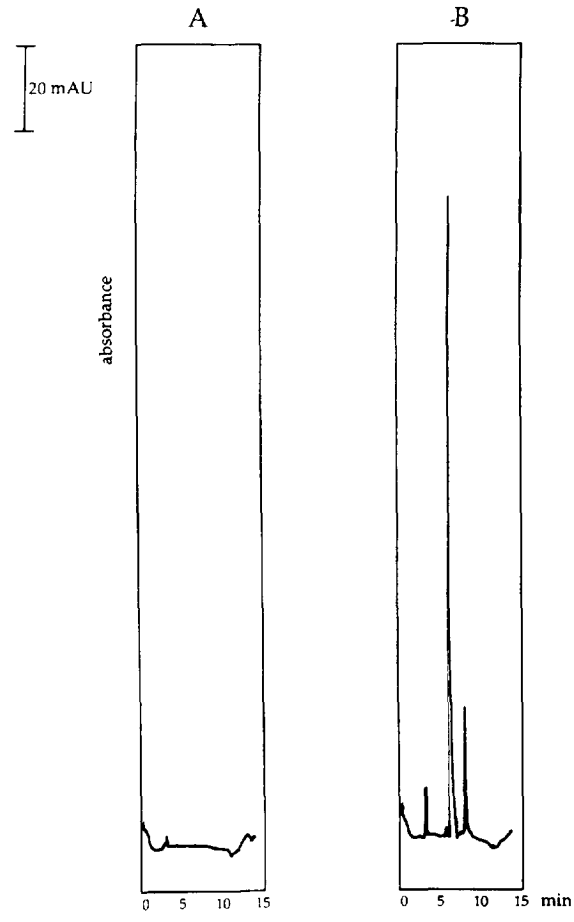


Fig. 2. Electropherograms of (A) pure water and (B) 50 μM ATP in water, analyzed by ED–CZE. Conditions: ED 20 s, −25 kV, CZE −25 kV; λ=200 nm.

3.3. Characterization and optimization

In order to get more insight into the ED–CZE system the influence of a number of parameters was investigated. First, the capillary inlet position in the acceptor compartment was examined. Best performance was achieved if the capillary inlet contacted the membrane, independent of the location on the membrane.

In comparison to electrokinetic injection ED differs with respect to the voltage applied. During ED a voltage is applied over the electrodialysis device (EDD) and the fused-silica capillary, whereas during electrokinetic injection the voltage is applied over the fused-silica capillary only. On the contrary, one of the similarities is the linear relationship between the injected/electrodialyzed amount of analyte and the applied voltage or electrodialysis time. In Fig. 3, the peak area of ATP obtained with ED is plotted as a function of time for different voltages. During these experiments the donor compartment contained 50 μM ATP in water, while the acceptor compartment was filled with pure water. Each data point consists of three measurements. For the lower curve (−5 kV) and for the middle curve (−15 kV), an almost linear relationship exists up to 60 s of electrodialysis time. For the upper curve (−25 kV), however, linearity does not hold above an electrodialysis time of 30 s. In the non-linear region the injected amount of analyte constitutes more than 1%

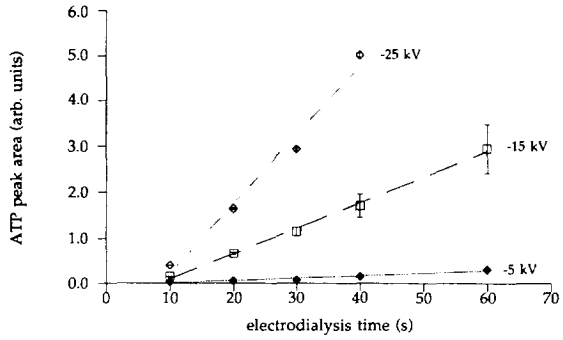


Fig. 3. ATP peak area as a function of the electrodialysis time for different voltages. Conditions: donor compartment, 50 μ*M* ATP in water; acceptor compartment, water; CZE voltage: −25 kV; λ= 200 nm.

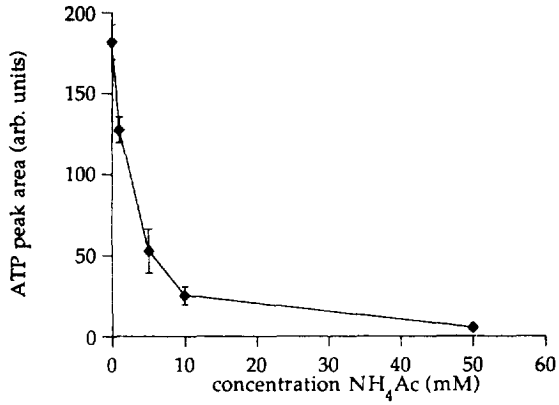


Fig. 4. Effect of the *donor* conductivity (NH_4Ac concentration) on the ATP peak area. Conditions: donor compartment, 100 μ*M* ATP in different matrices; acceptor compartment, water; ED, 20 s, −25 kV; CZE, −25 kV; λ=200 nm.

of the total capillary volume and leads to band broadening. Consequently, the peak width is significantly increased. Nevertheless, due to the low standard deviation also longer electrodialysis times can be used, which is favourable in determining low concentrations. In this paper, all further experiments were done at an electrodialysis voltage of −25 kV during 20 s which appeared an optimum of the system with respect to time and peak width.

Another similarity of electrokinetic injection and electrodialysis is the dependence on the sample conductivity. In order to investigate this, the donor compartment was filled with sample solutions, having the same ATP concentration but different ammonium acetate concentrations. The acceptor compartment was filled with pure water. Electrodialysis was performed by applying a voltage of −25 kV during 20 s after which CZE was carried out. Fig. 4 shows the relationship between the peak area of ATP and the ammonium acetate concentration in the sample. Each data point consists of three measurements. As a result of the difference in conductivity with respect to the sample zone field amplification or zone sharpening takes place, similar to the situation in electrokinetic injection of samples with a lower conductivity than that of the CZE buffer [30]. By raising the sample conductivity while keeping the ATP concentration constant, the peak area decreases, leading to a higher limit of detection (LOD). Only at a sample conductivity equal to that of the CZE buffer there is hardly any local difference in the electric field strength. Provided that the acceptor compartment contains pure water, the electrodialysis process is very similar to electrokinetic injection with respect to the sample conductivity.

Next to the donor compartment, the composition of the acceptor compartment can play a significant role during electrodialysis. As stated before, the electrodialysis process is similar to electrokinetic injection if the acceptor compartment contains pure water. But, a higher conductivity medium in the acceptor compartment affects the ED performance of ATP drastically, which is shown in Fig. 5. Each data point consists of three measurements. During all these experiments the donor compartment contained

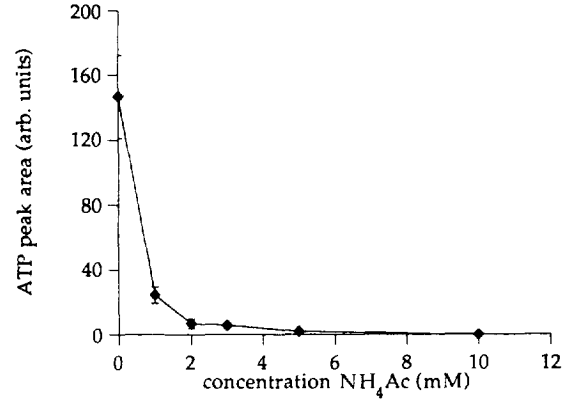


Fig. 5. Effect of the *acceptor* conductivity (NH_4Ac concentration) on the ATP peak area. Conditions: donor compartment, 100 μ*M* ATP in water; ED, 20 s, −25 kV; CZE, −25 kV; λ=200 nm.

100 μ*M* ATP in pure water. A slight increase of the ammonium acetate concentration in the acceptor compartment resulted in a significant decrease of the peak area. At 10 m*M* ammonium acetate, the ATP signal was even below the LOD. If not only the acceptor but also the donor compartment contained 10 m*M* ammonium acetate (next to ATP) the same result was obtained. Possibly, analyte stacking occurs if the acceptor compartment contains pure water. If, however, the acceptor compartment contains a higher conductivity medium the local electric field strength is normalized, resulting in a higher LOD. The low ionic strength of the acceptor may rise due to electrodialysis or electromigration of electrolytes from the donor compartment or the CZE buffer. In order to maintain quantitative reproducibility the acceptor compartment is filled with fresh water after every run. Furthermore, disposable membranes are used to prevent membrane fouling.

Separation of macromolecular compounds from the analyte, being one of the aims of electrodialysis, was investigated next. Therefore, the protein bovine albumin was added to the analyte solution at a concentration of 50 mg/ml which is similar to the human serum albumin (HSA) concentration in blood plasma. The total concentration of proteins in plasma is ca. 70 mg/ml [31]. Using a sample solution of pH 6, bovine albumin with a p*I* of 4.7 is negatively charged and will be introduced into the capillary without the use of a membrane. As the molecular mass of albumin (or HSA) is 67 000 and the membrane molecular mass cut-off was 30 000, albumin was retained in the donor compartment. In Fig. 6 the electropherograms are shown of a sample consisting of 10 μ*M* ATP and bovine albumin in water (left) and a blank solution containing only bovine albumin in water (right), analyzed with ED–CZE. The acceptor compartment was filled with pure water. Small peaks present in both electropherograms can be ascribed to impurities in the bovine albumin.

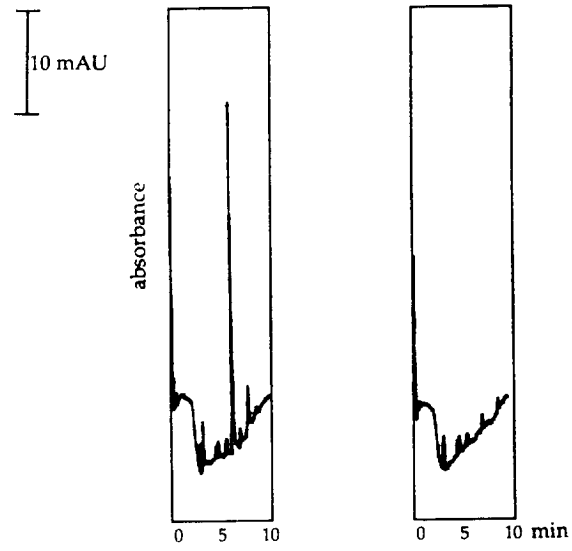


Fig. 6. Electropherograms of 10 μ*M* ATP and 50 mg/ml albumin in water (left) and 50 mg/ml albumin in water as the blank (right). Conditions: acceptor compartment, pure water; ED, 20 s, −25 kV; CZE, −25 kV; λ=200 nm.

3.4. Quantitative aspects

In order to use ED–CZE in quantitative analysis the system was validated with respect to linearity and sensitivity. For the investigation of the linearity, standard solutions of ATP ranging from 0.5 to 50 μ*M* were analyzed using ED–CZE. The method appeared to be linear over (at least) two decades with a correlation coefficient of 0.9993. Based on a signal-to-noise ratio of 3, a limit of detection (LOD) for ATP in water down to a concentration of 300 n*M* has been obtained. The LOD is significantly increased in other matrices than water, which is an inherent limitation in CZE. In order to lower the LOD in such situations, analytes can be selectively concentrated in the on-line mode by means of (transient) isotachophoresis [32–36] after electrodialysis.

3.5. Applications

3.5.1. Analysis of inositol phosphates in fermentation broth

The developed method has been applied to the determination of inositol phosphates in fermentation broth which requires a sample pretreatment in order to prevent capillary clogging and/or wall adsorption by yeast cells. Therefore, fermentation broth was spiked to a concentration of 100 μ*M* inositol hexakis-(IP_6), tetrakis-(IP_4), tris-(IP_3), bis-(IP_2) and monophosphate (IP_1) and subsequently analyzed with ED–CZE. The result is depicted in Fig. 7. IP_6

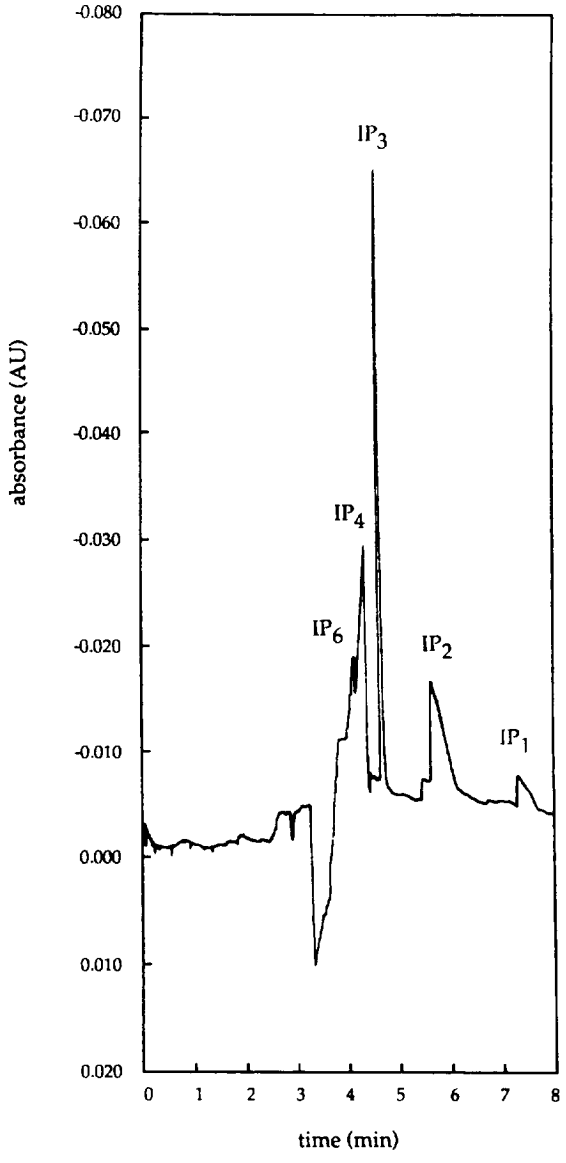


Fig. 7. Determination of inositol phosphates in fermentation broth by ED–CZE. Conditions: CZE buffer, 0.5 m*M* NDSA, 30 m*M* acetic acid pH 3, 0.005% HPMC; indirect UV detection at 214 nm.

and IP_4 could not be base-line separated due to the home-built instrumental set-up which complicated a further increase of the electric field strength. Nevertheless, it can be concluded that the electropherogram obtained by ED–CZE is very similar to the one obtained after centrifugation of the sample, followed by electrokinetic injection of the supernatant and CZE (not shown). However, off-line centrifugation–electrokinetic injection takes more than 5 min whereas on-line electrodialysis takes only 20 s.

3.5.2. Determination of ATP in plasma

Blank plasma was spiked with ATP to a concentration of 100 μ*M* and analyzed with ED–CZE. Next to the high amount of proteins, plasma contains, among others, chloride, phosphate, sodium, carbohydrates, fatty acids and lipids [31]. In order to enhance the selectivity of the method, UV detection was performed at 259 nm. Fig. 8 shows the electropherograms of ATP in plasma (Fig. 8A) and blank plasma (Fig. 8B). Although the electropherograms show that bioanalysis is possible by ED–CZE, it must be stated that the sensitivity for real-life samples has to be improved. The low sensitivity is the result of the relatively high conductivity of plasma; analysis of a 1:10 diluted plasma sample showed a similar analyte signal.

As the sensitivity of the method is limited for high-conductivity samples, future research will be devoted to on-line, selectively concentrating techniques between ED and CZE. A minor disadvantage of the ED–CZE system is that samples containing analytes bound to macromolecular compounds, e.g. plasma proteins cannot be analyzed as such. In that case, an extra sample pretreatment step must be introduced to release the analyte from the macromolecular compounds.

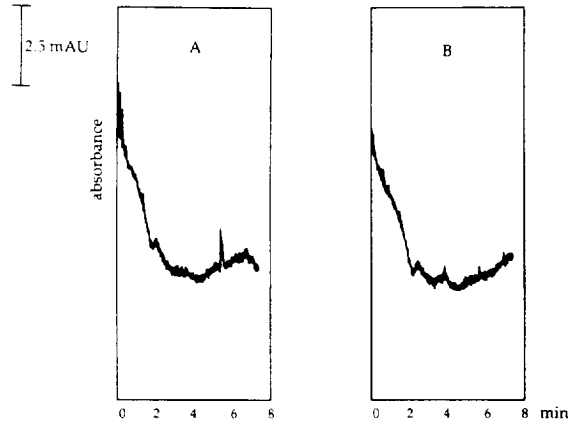


Fig. 8. Bioanalysis of ATP by ED–CZE. (A) 100 μ*M* ATP in plasma, (B) plasma. Conditions: acceptor compartment, pure water; ED, 20 s, −25 kV; CZE, −25 kV; λ=259 nm.

4. Conclusions

Electrodialysis has been coupled on-line to capillary zone electrophoresis. Macromolecular compounds in the sample, like albumin or yeast cells, are effectively retained by the membrane whereas small negatively charged ions are introduced into the capillary in only ca. 20 s. Similar to electrokinetic injection, the sample conductivity has a negative effect on the amount of analyte electrodialyzed into the capillary. Sofar, bioanalysis can be performed only at relatively high analyte concentrations. Future research will be focused on sensitivity enhancement in ED of high-conductivity samples.

Acknowledgments

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